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| | | |
|---|---|---|
| 1. | Your reference | 4-32567P1 |
| 2. | Patent application number *(The Patent Office will fill in this part)* | 04 JUL 2002   0215509.1 |
| 3. | Full name, address and postcode of the or of each applicant *(underline all surnames)* | NOVARTIS AG<br>LICHTSTRASSE 35<br>4056 BASEL<br>SWITZERLAND |
| | Patent ADP number *(if you know it)* | 07125487005 |
| | If the applicant is a corporate body, give the country/state of its incorporation | SWITZERLAND |
| 4. | Title of invention | ~~Organic compounds~~ MARKER GENES |
| 5. | Name of your agent *(If you have one)*<br>"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* | B.A. YORKE & CO.<br>CHARTERED PATENT AGENTS<br>COOMB HOUSE, 7 ST. JOHN'S ROAD<br>ISLEWORTH<br>MIDDLESEX TW7 6NH |
| | Patents ADP number *(if you know it)* | 1800001 |

| | | Country | Priority application number *(if you know it)* | Date of filing (day/month/year) |
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| 6. | If you are declaring priority from one ore more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number | | | |

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| Priority documents | |
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| Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* | |
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11. I/We request the grant of a patent on the basis of this application

Signature B.A. Yorke & Co

Date

**B.A. Yorke & Co.** **04 July 2002**

12. Name and daytime telephone number of person to contact in the United Kingdom

**Mrs. E. Cheetham**
**020 8560 5847**

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# Marker genes

## Field of the Invention

The present invention relates to methods for the monitoring, prognosis, diagnostic and/or treatment of renal disorders, i.e. renal diseases, injuries or toxicities. In particular, the invention relates to the use of gene expression analysis to determine renal disorders and/or to help choosing or monitoring the efficacy of various treatments for renal disorders.

## Background

The study of genetic and genomic factors at the DNA/RNA level involved in an individual's response to a foreign compound or drug permits the selection of safe agents (e.g., drugs) for prophylactic or therapeutic treatments. Agents or modulators which have a stimulatory or inhibitory effect on expression of a marker of the invention can be monitored in individuals to assess renal toxicity in the patient. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly, the level of expression of a marker of the invention in an individual can be determined to thereby select appropriate safe agent(s) for therapeutic or prophylactic treatment of the individual.

Pharmacogenetic deals with clinically significant variations in the efficacy or toxicity of drugs due to variations in drug disposition and action in individuals. See, e.g., Linder, Clin. Chem., 43(2):254-266 (1997). In general, two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body are referred to as "altered drug action". Genetic conditions transmitted as single factors altering the way the body acts on drugs are referred to as "altered drug metabolism". These pharmacogenetic conditions can occur either as rare defects or as common polymorphisms.

The level of expression, or the level of function, of a marker in an individual can be determined to thereby select appropriate agent for therapeutic or prophylactic treatment of the individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure, and thus enhance therapeutic or prophylactic efficiency when treating a subject with a modulator of expression of a marker. **Calbindin D-28k** is a calcium-binding protein member of the large EF-hand family. It is present in all classes of vertebrates and in a wide range of tissues. Calbindin D-28k is postulated to

function as a calcium transport molecule that facilitates the diffusion of calcium through the cell and serves as an intracellular calcium buffer maintaining the ionized calcium below toxic levels (1).

Several researchers have shown that in the kidney highest amounts of calbindin D-28k are localized in the distal tubule, which correlates with the role of the distal tubule as the site of calcium absorption (2, 3). Furthermore it has been shown that the decreased expression of calbindin D-28k with age may contribute to the age-related decrease in $Ca^{++}$ transport in intestine and kidney(4). It is reported in the literature that cyclosporine A-induced decrease in rat renal calbindin-D28kDa protein is a consequence of a decrease in its mRNA (5, 6). This statement implies that the calbindin-D28k mRNA levels can be monitored by PCR, which reflects protein levels(7). These observations confirm the hypothesis that calbindin D-28k has an important role in the re-absorption process taking place in the kidney.

**Kidney injury molecule-1** (KIM-1) mRNA and protein are expressed at a low level in normal kidney but are increased dramatically in postischemic kidney. KIM-1 may play an important role in the restoration of the morphological integrity and function to kidney(8).

**Osteopontin** (OPN) is a highly acidic phosphoprotein containing an arginine-glycine-aspartic acid (RGD) cell adhesion motif. High OPN expression is regulated by the vitamin $D_3$ active form (9) and has been found in tissues with high cell turnover. OPN up-regulation has been demonstrated in several models of renal injury, suggesting a possible role in tissue remodeling and repair (10). However, its exact function in the kidney remains unknown.

**Epidermal growth factor** (EGF) is a small polypeptide belonging to a class of molecules that can mediate cell growth, differentiation, and acute phase responses. EGF mRNA is transcribed primarily in cells of the salivary gland and the kidney. In a variety of experimentally induced forms of acute renal failure, the mRNA and protein levels for kidney EGF fall markedly and remain low for a prolonged period (11). The epidermal growth factor (EGF) is important epithelial mitogens. In addition EGF receptor levels are known to play a central role in density dependent growth regulation of normal rat kidney fibroblasts (12). EGF is involved in the endogenous tissue repair after acute renal injury. This growth factor accelerates with the recovery of renal function and the anatomical restoration of tubular integrity when given exogenously to laboratory animals with experimental acute renal failure (13). The renal epidermal growth factor (EGF) mRNA level is decreased very early in cyclosporine A treated rat kidneys (14).

**Clusterin** is a ubiquitous glycoprotein induced in many organs, including the kidney, at times of tissue injury and/or remodeling. Clusterin is a soluble complement regulatory protein that binds to C5b-7 and inhibits generation of membrane attack complex, C5b-9. Glomerular deposition of clusterin has been observed in human and experimental membranous nephropathy in association with C5b-9 and immune deposits (15). It is speculated that clusterin preserves cell interactions that are otherwise perturbed by renal insults (16). Interestingly it is reported in the literature that CsA increases clusterin mRNA levels in the rat kidney (17).

Although some of the above markers are speculated to be associated with nephropathies, these markers have not been actually used as diagnostics for determining renal disorders, and their levels of expression have never been correlated to various renal disorder status. In the field of renal nephropathies, there is however a need to allow election of appropriate agent for therapeutic or prophylactic treatment, prediction of an individual's drug responsiveness phenotype, and selection of dosing or drug in order to avoid adverse reactions or therapeutic failure.

In addition, so far only no polymorphism in a gene has been described and correlated with clinical effects of renal disorders. A major question remains in this field whether more of such polymorphisms exist and, if so, whether these can be correlated with drug activity and/or drug side effects.

## Summary of the Invention

The present invention relates to a method for determining renal toxicity in an individual comprising the steps of (i) obtaining a body sample from an individual, (ii) determining from the body sample the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value, and (iii) comparing the first set of value with a second set of value corresponding to the level of gene expression assessed for the same gene(s) and under identical condition as for step b) in a body sample of an individual not subject to renal toxicity, wherein the first value lower than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the individual of step a) is having, developing or sensitive to renal toxicity, and/or wherein the first value greater than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the individual is having, developing or sensitive to renal toxicity.

The present invention further covers a test for use in determining whether a renal toxicity in an individual responds to therapy comprising the steps of, performing steps a), b) and c) of the invention for a body sample obtained from an individual treated against renal toxicity with a pharmaceutically acceptable agent and determining the responsiveness of the individual to drug therapy.

The present invention further covers another test for use in determining whether a kidney toxicity in an individual responds to therapy treatment comprising the steps of, performing steps a), b) and c) of the invention for a body sample obtained from an individual treated against renal toxicity with a pharmaceutically acceptable agent and determining the responsiveness of the individual to drug therapy.

In another aspect, the invention covers a method for treating renal toxicity in an individual comprising the step of administering to said individual a therapeutically effective amount of a modulating compound that modulates in the kidney the synthesis, expression or activity of one or more of the genes or gene expression products of the group of genes Calbindin-D28k, KIM-1, OPN, EGF and/or Clusterin, so that at least one symptom of renal toxicity is ameliorated.

In another aspect, the invention covers a method for identifying candidate agents for use in the treatment of renal toxicity comprising the steps of (i) contacting a sample of a kidney tissue subject to toxicity with a candidate agent, (ii) determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value, and (iii) comparing the first set of value with a second set of value corresponding to the level of gene expression assessed for the same gene(s) and under identical condition as for step b) in a kidney tissue subject to toxicity not induced by the candidate agent, wherein a first value substantially greater than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the candidate agent is ameliorating renal toxicity symptoms, and/or wherein a first value substantially lower than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the candidate agent is ameliorating renal toxicity symptoms.

In a further aspect, the invention covers a method for identifying candidate agents that do not provoke or induce renal toxicity comprising the steps of (i) contacting a sample of a kidney tissue not subject to toxicity with a candidate agent, (ii) determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value, and

(iii) comparing the first set of value(s) with a second set of value corresponding to the level of gene expression assessed for the same gene(s) and under identical condition as for step b) in a kidney tissue not subject to toxicity, wherein a first value equal or higher than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the candidate agent does not provoke or induce renal toxicity, and/or wherein a first value equal or lower than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the candidate agent does not provoke or induce renal toxicity.

In a further aspect, the invention covers a method for comparing renal cytotoxic potentials of two drug candidates comprising the steps of (i) contacting a sample of a kidney tissue not subject to toxicity with a first drug candidate, and determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value, (ii) contacting a sample of a kidney tissue not subject to toxicity with a second drug candidate, and determining from the kidney tissue level(s) of gene expression(s) corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a second set of value, and (iii) comparing the first set of value to the second set of value, wherein if the first value is substantially greater than the second value for Calbindin-D28K and/or EGF gene expression this is an indication that the seconddrug candidate is less cytotoxic to the kidney than the first drug candidate, and/or wherein if the first value is substantially lower than the second value for KIM-1, Osteopontin and/or Clusterin gene expression this is an indication that thesecond drug candidate is less cytotoxic to the kidney than the first drug candidate.

In a last aspect, the invention provides the use of some polymorphism in a gene for the diagnostic of renal toxicity, wherein the gene are chosen from Calbindin-D28k, KIM-1, OPN, EGF and Clusterin.

**Brief Description of the Drawings**

Figure 1: Represents measurements by Western Blotting of clusterin protein plasma levels after treatment with 1: non-nephrotoxic compound (A), and 2: nephrotoxic compounds (B, C, D).

Figure 2: Represents the evolution of the expression changes of gene markers linked to renal pathology status. The pathology scoring is defined as follows :1 = minimal, very few; 2 = slight, few; 3 = moderate, moderate number; 4 = marked, many; 5 = severe, extensive number.

Figure 3: Represents the occurrence of renal gene expression changes versus classical biochemical endpoint (creatinine levels). The pathology scoring is defined as follows :1 = minimal, very few; 2 = slight, few; 3 = moderate, moderate number; 4 = marked, many; 5 = severe, extensive number.

Figure 4: Represents relative fold expression-changes of marker genes in kidney of rat treated with two test compounds (TC1 and TC2) and Neoral®. A.U.: Arbitrary units.

**Detailed Description of the Invention**

As used herein the expression "renal toxicity" or "renal injury" or similarly "kidney disorder" shall all mean a renal or kidney failure or dysfunction either sudden (acute) or slowly declining over time (chronic), that may be triggered by a number of disease or disorder processes, including (but not limited to) for acute renal toxicity: sepsis (infection), shock, trauma, kidney stones, kidney infection, drug toxicity, poisons or toxins, or after injection with an iodinated contrast dye (adverse effect); and for chronic renal toxicity: long-standing hypertension, diabetes, congestive heart failure, lupus, or sickle cell anemia. Both forms of renal failure result in a life-threatening metabolic derangement.

The expression "body samples" shall include but is not limited to biopsies, preferably of the kidney, and body fluids such as blood, plasma, serum, lymph, cerebro-spinal fluid, cystic fluid, ascites, urine, stool and bile, for instance. One advantage of the present invention is that one marker can be particularly well monitored in body fluids, such as plasma. For instance, clusterin's level of expression can be particularly well determined in plasma.

As used herein the term "Individual" shall mean a human person, an animal or a population or pool of individuals.

As used herein, the term "candidate agent" or "drug candidate" can be natural or synthetic molecules such as proteins or fragments thereof, antibodies, small molecule inhibitors or agonists, nucleic acid molecules, e.g., antisense nucleotides, ribozymes, double-stranded RNAs, organic and inorganic compounds and the like.

mRNA expression levels that are expressed in absolute values represent the number of molecules for a given gene calculated according to a standard curve. To perform quantitative measurements serial dilutions of a cDNA (standard) are included in each experiment in order to construct a standard curve necessary for the accurate mRNA quantitation. The absolute values (number of molecules) are given after extrapolation from the standard curve.

As used herein each marker referred to as "Calbindin-D28k", "KIM-1", "OPN", "EGF" or "Clusterin" encompass the gene or gene product (including mRNA and protein) that are substantially similar to the markers identified below in table 1.

In its broadest sense, the term "substantially similar", when used herein with respect to a nucleotide sequence, means a nucleotide sequence corresponding to a reference nucleotide sequence, wherein the corresponding sequence encodes a polypeptide having substantially the same structure and function as the polypeptide encoded by the reference nucleotide sequence, e.g. where only changes in amino acids not affecting the polypeptide function occur. Desirably the substantially similar nucleotide sequence encodes the polypeptide encoded by the reference nucleotide sequence. The percentage of identity between the substantially similar nucleotide sequence and the reference nucleotide sequence desirably is at least 80%, more desirably at least 85%, preferably at least 90%, more preferably at least 95%, still more preferably at least 99%. Sequence comparisons are carried out using a Smith-Waterman sequence alignment algorithm (see e.g. Waterman, M.S. Introduction to Computational Biology: Maps, sequences and genomes. Chapman & Hall. London: 1995. ISBN 0-412-99391-0). The localS program, version 1.16, is used with following parameters: match: 1, mismatch penalty: 0.33, open-gap penalty: 2, extended-gap penalty: 2.

A nucleotide sequence "substantially similar" to reference nucleotide sequence can also hybridize to the reference nucleotide sequence in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 2X SSC, 0.1% SDS at 50°C, more desirably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 1X SSC, 0.1% SDS at 50°C, more desirably still in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.5X SSC, 0.1% SDS at 50°C, preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 50°C, more preferably in 7% sodium dodecyl sulfate (SDS), 0.5 M $NaPO_4$, 1 mM EDTA at 50°C with washing in 0.1X SSC, 0.1% SDS at 65°C, yet still encodes a functionally equivalent gene product.

The present invention provides a plurality of markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) that together or alone, are or can be used as markers of renal toxicity. In particularly useful embodiments, a plurality of these markers can be selected and their mRNA expression monitored simultaneously to provide expression profiles for use in various aspects.

In a preferred embodiment of the present methods, at least 2 or 3, preferably 5 markers selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, can be used for determination of their gene expression profiles.

As each marker can be linked to different renal pathological findings, it is possible to identify gene expression profiles of such markers that are particularly linked to a renal pathology. For instance, the cabindin-D28k mRNA level is used as an early marker for calcium disturbance predictor for mineralization. The KIM-1 mRNA level is a marker for general kidney insult. The OPN mRNA level is an early marker for macrophage infiltration often associated with kidney toxicity and a marker for tissue remodeling upon renal injury. The EGF mRNA level is an early marker for general kidney toxicity. The clusterin mRNA level is an early marker for immune-mediated kidney toxicity.

In a further preferred embodiment of the present methods, mRNA expression is assessed in the body samples or kidney tissues by techniques selected from the group consisting of Northern blot analysis, reverse transcription PCR, real time quantitative PCR, NASBA, TMA, or any other available amplification technology.

In another preferred embodiment of the present methods, the level of gene expression can alternatively be assessed by detecting the presence of a protein corresponding to the gene expression product.

It must be noted that mRNA expression levels expressed in absolute values in the present invention (see below) for Calbindin-D28k, KIM-1, OPN, EGF and Clusterin are generally found in most population's type or specie. These values may however possibly vary for each population's type or specie. It may therefore be necessary to determine again for each marker the standard gene expression level for a targeted population's type or specie which is not subject to renal toxicity, above or under which, as appropriate, renal toxicity symptoms can be found.

In a first particular aspect of the invention, a method is provided for determining renal toxicity in an individual, the steps comprise (a) obtaining a body sample from an individual; (b) determining from the body sample the level of gene expression

corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value; (c) and comparing the first set of value with a second set of valuecorresponding to the level of gene expression, assessed for the same gene(s) and under identical condition as for step b) in a body sample of an individual not subject to renal toxicity, wherein the first value lower than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the individual of step a) is having, developing or sensitive to renal toxicity, and/or wherein the first value greater than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the individual of step a) is having, developing or sensitive to renal toxicity.

In a preferred embodiment of such method, mRNA expression levels in the body sample of the individual of step a), of Calbindin-D28k below 5.30E+08, of KIM-1 above 1.50E+07, of EGF below 2.80E+08, of Osteopontin above 1.40E+08, and/or of Clusterin above 1.90E+09, indicates that such individual is having, developing or sensitive to renal toxicity, wherein mRNA expression is determined in absolute value. These values may however possibly vary for each population's type or specie.

In a second particular aspect of the invention, the expression profiles of one or a plurality of these markers could provide valuable molecular tools for examining the molecular basis of drug responsiveness in renal toxicity and for evaluating the efficacy of drugs for treating renal toxicity or their side effects on the kidney. Changes in the expression profile from a baseline profile while the cells are exposed to various modifying conditions, such as contact with a drug or other active molecules can be used as an indication of such effects.

Therefore, the invention provides a test for use in determining whether a renal toxicity in a patient will respond to therapy comprising the steps of, performing steps a), b) and c) of the method above for body samples obtained respectively from an individual treated against renal toxicity with a pharmaceutically acceptable agent and an individual not subject to renal toxicity, and determining the responsiveness to drug therapy.

Monitoring the influence of agents (e.g., drug compounds) on the level of expression of a marker of the invention can be advantageously applied in clinical trials. For example, the effectiveness of an agent to affect marker expression can be monitored in clinical trials of subjects receiving treatment for renal disease or toxicity. In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g., an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) comprising the steps of: (i)

obtaining a pre-administration sample from a subject prior to administration of the agent; (ii) detecting the level of expression of one or more selected markers of the invention in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression of the marker(s) in the post-administration samples; (v) comparing the level of expression of the marker(s) in the pre-administration sample with the level of expression of the marker(s) in the post-administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, modified administration of the agent can be desirable to increase expression of the marker(s) to higher levels than detected, i.e., to increase the effectiveness of the agent. Alternatively, increased/decreased administration of the agent can be desirable to increase/decrease the effectiveness of the agent, respectively.

In a third particular aspect of the present invention, a method is provided for both prophylactic and therapeutic methods of treating a subject having, or at risk of having, a kidney disorder or renal toxicity. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the kidney disorder, such that development of the kidney disorder is prevented or delayed in its progression. Examples of suitable therapeutic agents include, but are not limited to, antisense nucleotides, ribozymes, double-stranded RNAs, ligands, small molecules and antagonists as described more in detail below.

In a particular embodiment, the invention provides a method for treating or preventing renal toxicity in an individual comprising the step of administering to said individual a therapeutically effective amount of a modulating compound that modulates in the kidney the synthesis, expression or activity of one or more of the genes or gene expression products of the group of genes Calbindin-D28k, KIM-1, OPN, EGF and/or Clusterin, so that at least one symptom of renal toxicity is ameliorated. Preferably after treatment of an individual with the modulating compound the gene mRNA expression in a body sample of such individual, of Calbindin-D28k above to 5.30E+08, of KIM-1 below 1.50E+07, of EGF above 2.80E+08, of Osteopontin below 1.40E+08, and/or of Clusterin below 1.90E+09, indicates that at least one symptom of renal toxicity is ameliorated, wherein gene mRNA expression is determined in absolute value. These values may however possibly vary for each population's type or specie.

In a fourth particular aspect of the invention, by virtue of the differential expression of the markers, it is possible to utilize these markers to enhance the certainty of prediction of whether a particular drug treatment in a patient will not be toxic to the kidney. Therefore, the invention provides a method for identifying candidate agents for use in the

treatment of renal toxicity comprising the steps of: a) contacting a sample of a kidney tissue subject to toxicity with a candidate agent; b) determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value; and c) comparing the first set of value with a second set of value corresponding to the level of gene expression, assessed for the same gene(s) and under identical condition as for step b) in a kidney tissue subject to toxicity not induced by the candidate agent, wherein a first value substantially equal or greater than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the candidate agent is ameliorating renal toxicity symptoms, and/or wherein a first value substantially equal or lower than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the candidate agent is ameliorating renal toxicity symptoms.

In a preferred embodiment, mRNA gene expression in kidney tissue subject to toxicity, of Calbindin-D28k above 5.30E+08, of KIM-1 below 1.50E+07, of EGF above 2.80E+08, of Osteopontin below 1.40E+08, and/or of Clusterin below 1.90E+09, is an indication that the candidate agent is ameliorating renal toxicity, wherein mRNA gene expression is determined in absolute value. These values may however possibly vary for each population's type or specie.

In a fifth particular aspect of the present invention, a method is provided for identifying candidate agents that do not provoke or induce renal toxicity comprising the steps of: a) contacting a sample of a kidney tissue not subject to toxicity with a candidate agent; b) determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value; and c) comparing the first set of value with a second set of value corresponding to the level of gene expression assessed for the same gene(s) and under identical condition as for step b) in a kidney tissue not subject to toxicity, wherein a first value substantially equal or greater than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the candidate agent does not provoke or induce renal toxicity, and/or wherein a first value substantially equal or lower than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the candidate agent does not provoke or induce renal toxicity.

In a preferred embodiment, mRNA expression levels determined in the kidney tissue not subject to toxicity, of Calbindin-D28k above 5.30E+08, of KIM-1 below 1.50E+07, of EGF above 2.80E+08, of Osteopontin below 1.40E+08, and/or of Clusterin

below 1.90E+09, is an indication that the candidate agent does not provoke or induce renal toxicity, wherein mRNA expression is determined in absolute value. These values may however possibly vary for each population's type or specie.

In a sixth particular aspect of the present invention, a method is provided for comparing renal cytotoxic potentials of two drug candidates comprising the steps of: a) contacting a sample of a kidney tissue not subject to toxicity with a first drug candidate, and determining from the kidney tissue level(s) of gene expression(s) corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first value; and b) contacting a sample of a kidney tissue not subject to toxicity with a second drug candidate, and determining from the kidney tissue level(s) of gene expression(s) corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a second value; and c) comparing the first value with the second value, wherein if the first value is substantially lower than the second value for Calbindin-D28K and/or EGF gene expression(s) this is an indication that the second drug candidate is less cytotoxic to the kidney than the second drug candidate, and/or wherein if the first value is substantially higher than the second value for KIM-1, Osteopontin and/or Clusterin gene expression(s) this is an indication that the second drug candidate is less cytotoxic to the kidney than the second drug candidate.

One particular advantage of the last three methods above, i.e., methods (i) for identifying candidate agents, (ii) for comparing renal cytotoxic potentials of two drug candidates and (iii) for identifying candidate agents that do not provoke or induce renal toxicity, is that they an be performed *in-vitro*. The kidney tissues that are used are preferably obtained from a cultured kidney tissue or cells that have been contacted with a cytotoxic agent. The kidney tissue can also be a kidney sample of an individual subject to renal toxicity, but this may limit broad *in-vitro* applications of such methods.

Cultured kidney tissue or cells may be advantageously based on an in vivo animal model that mimics human cellular and tissues disorders, preferably of the kidney. It may also be a single or collection of kidney cells such as the human kidney epithelial 293Tcells or a human embryonic kidney cell line, for instance. The cytotoxic agent may be any molecule having a known toxicity towards kidney, and may advantageously be selected from many examples that include: cyclosporin, cisplatin, aminoglycosides, sulfonamides, tacrolimus, trimethadione, etc. The kidney is particularly susceptible to the nephrotoxic action of drugs, because of its functional properties, including: a) the high volume of renal blood flow, which brings large amounts of toxin; b) the large area in contact with the drug, either in the glomerulus or the tubule epithelium, which enables toxin interaction or

uptake; c) the kidney's ability to transfer active substances, which provides specific transfer mechanisms that mediate cellular uptake; d) drug breakdown, which may occur in renal tubules and lead to the formation of toxic metabolites from non-toxic parent substances; e) the kidney's concentrating mechanisms, which can increase urinary and interstitial concentrations of non-absorbed products; f) the high metabolic rate of tubule cells required for normal function, which is subject to perturbation.

The concentration of cyclosporin (e.g. Neoral®) can range from 10E-11 to 10E-5 M in the case of *in vitro* studies. These values may however possibly vary for each population's cell type or culture conditions.

Measurement methods

A particularly useful method for detecting the level of mRNA transcripts obtained from the markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) involves hybridization of labeled mRNA to an ordered array of oligonucleotides. Such a method allows the levels of transcription of a plurality of these genes to be determined simultaneously to generate gene expression profiles or patterns. The gene expression profile derived from the sample obtained from the subject can, in another embodiment, be compared with the gene expression profile derived form the sample obtained from the disease-free subject, and thereby determine whether the subject has or is at risk of developing renal disease or toxicity.

The gene expressions of the markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) can be preferably assessed in the form of a kit using RT-PCR, a high throughput technology: The well-known technique RT-PCR reaction exploits the 5′ nuclease activity of AmpliTaq Gold DNAPolymerase to cleave a TaqMan probe during PCR. The probe consists of an oligonucleotide (usually ≈20 mer) with a 5'-reporter dyeand a 3'-quencher dye. The fluorescent reporter dye, such as FAM (6-carboxyfluorescein), is covalently linked to the 5' end of the oligonucleotide. The reporter is quenched by TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine) attached via a linker arm that is located at the 3' end.

Oligonucleotide probes used for each marker (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) should derive from the nucleotide sequence of the gene of such marker, the selection of the appropriate oligonucleotide sequence being now a matter of standard routine technique for one skilled in the art. The following table 1 gives various access codes of the Genbank database for marker sequences in humans, rat and/or mouse.

Table 1: Sequences of the marker genes

| Gene name | Calbindin-D28k (CALB1) | KIM-1 | Osteopontin (Uropontin, SPP-1) | Clusterin (TRMP-2; Apolipoprot. J) | EGF |
|---|---|---|---|---|---|
| Genbank # | **Rat:** M31178 | **Rat:** AF035963 | **Rat:** AB001382 M99252 | **Rat:** U02391 M64723 | **Rat:** AF187818 |
| Genbank # | **Human:** NM_004929AC004 612 AF049895 AF068862 AF070717 BC006478 M19878 M19879 X06661 | **Human:** AL159977 AC073225.5 AC025449.6 AF165926 AL449103 | **Human:** AF052124 D14813 J04765 M83248 U20758 X13694 | **Human:** AF311103 J02908 L00974 M25915 M63379 M64722 M74816 X14723 | **Human:** J02548 X04571 |
| Genbank # | **Mouse:** AK002635 AK005081 AK005243 D26352 D26353 D26354 D26355 D26356 D26357 M21531 M23663 | **Mouse:** AI662116 | **Mouse:** J04806 M38399 S78177 X13986 X14882 X16151 X51834 | **Mouse:** AF182509 D14077 L05670 L08235 S70244 | **Mouse:** J00380 U69534 V00741 X08047 |

The protein expressions of the markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) that are secreted by both normal and disease cells can be also analyzed and are of value in the methods of this invention. Supernatants can be isolated and MWT-CO filters can be used to simplify the mixture of proteins. The proteins can then be digested with trypsin. The tryptic peptides may then be loaded onto a microcapillary HPLC column where they are separated, and eluted directly into an ion trap mass spectrometer, through a custom-made electrospray ionization source. Throughout the gradient, sequence data can be acquired through fragmentation of the four most intense ions (peptides) that elute off the column, while dynamically excluding those that have already been fragmented. In this way, the sequence data from multiple scans can be obtained, corresponding to approximately 50 to 200 different proteins in the sample. These data are searched against databases using correlation analysis tools, such as MS-Tag, to identify the protein expressions of the markers in the supernatants.

Expression of the protein encoded by the markers can also be detected by a probe which is detectably labeled, or which can be subsequently labeled. Generally, the probe is an antibody that recognizes the expressed protein.

As used herein, the term antibody includes, but is not limited to, polyclonal antibodies, monoclonal antibodies, humanized or chimeric antibodies, and biologically functional antibody fragments sufficient for binding of the antibody fragment to the protein.

The extent to which the known proteins are expressed in the sample is then determined by immunoassay methods that utilize the antibodies described above. Such immunoassay methods include, but are not limited to, dot blotting, western blotting, competitive and noncompetitive protein binding assays, enzyme-linked immunosorbant assays (ELISA), immunohistochemistry, fluorescence activated cell sorting (FACS), and others commonly used and widely described in scientific and patent literature, and many employed commercially.

Alternatively, marker proteins (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) can be separated by two-dimensional gel electrophoresis systems. Two-dimensional gel electrophoresis is well known in the art and typically involves iso-electric focusing along a first dimension followed by SDS-PAGE electrophoresis along a second dimension. The resulting electropherograms can be analyzed by numerous techniques, including mass spectrometric techniques, western blotting and immunoblot analysis using polyclonal and monoclonal antibodies, and internal and N-terminal micro-sequencing.

<u>Drug screening methods</u>

In addition to the drug screening methods described above, cell-free assays can also be used to identify compounds which are capable of interacting with proteins encoded by the markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin), to alter the activity of the protein or its binding partner. Cell-free assays can also be used to identify compounds, which modulate the interaction between the encoded protein and its binding partner such as a target peptide.

In one embodiment, cell-free assays for identifying such compounds comprise a reaction mixture containing a marker protein (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) and a test compound or a library of test compounds in the presence or absence of the binding partner, e.g., a biologically inactive target peptide, or a small molecule. Interaction between molecules can also be assessed by using real-time BIA (Biomolecular Interaction Analysis, Pharmacia Biosensor (AB) which detects surface plasmon

resonance, an optical phenomenon. Formation of a complex between the protein and its binding partner can be detected by using detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled protein or its binding partner, by immunoassay or by chromatographic detection.

Transcript array

In a preferred embodiment the present invention makes use of "oligonucleotide arrays" (also called herein "microarrays"). Microarrays can be employed for analyzing the transcriptional state in a cell, and especially for measuring the transcriptional states of kidney cells.

In one embodiment, transcript arrays are produced by hybridizing detectably labeled polynucleotides representing the mRNA transcripts present in a cell (e.g., fluorescently labeled cDNA synthesized from total cell mRNA or labled cRNA.) to a microarray. A microarray in the present invention is a surface with an ordered array of binding (e.g., hybridization) sites for products of at least one of the marker genes (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin). Microarrays can be made in a number of ways. However produced, microarrays share certain characteristics: The arrays are reproducible, allowing multiple copies of a given array to be produced and easily compared with each other. Preferably the microarrays are small, usually smaller than 5 cm.sup.2, and they are made from materials that are stable under binding (e.g. nucleic acid hybridization) conditions. A given binding site or unique set of binding sites in the microarray will specifically bind the product of a single gene in the cell. Although there may be more than one physical binding site (hereinafter "site") per specific mRNA, for the sake of clarity the discussion below will assume that there is a single site. In a specific embodiment, positionally addressable arrays containing affixed nucleic acids of known sequence at each location are used.

It will be appreciated that when cDNA complementary to the RNA of a cell is made and hybridized to a microarray under suitable hybridization conditions, the level of hybridization to the site in the array corresponding to any particular gene will reflect the prevalence in the cell of mRNA transcribed from that gene. For example, when detectably labeled (e.g., with a fluorophore) cDNA or cRNA complementary to the total cellular mRNA is hybridized to a microarray, the site on the array corresponding to a gene (i.e., capable of specifically binding the product of the gene) that is not transcribed in the cell will have little or no signal (e.g., fluorescent signal), and a gene for which the encoded mRNA is prevalent will have a relatively strong signal.

Databases

This invention also provides a process for preparing a database comprising gene expression profiles for at least one of the markers set forth in this invention (table 1). For example, the gene expression profiles for each marker can be stored in a digital storage medium such that a data processing system for standardized representation of the markers profiles, alone or in combination, that identify a particular renal disease or toxicity cell is compiled.

Alternative computer systems and methods for implementing the analytic methods of this invention will be apparent to one of skill in the art and are intended to be comprehended within the accompanying claims. In particular, the accompanying claims are intended to include the alternative program structures for implementing the methods of this invention that will be readily apparent to one of skill in the art.

Antisense molecules

In another embodiment, activity of a target RNA (preferable mRNA) species, specifically its rate of translation, can be controllably inhibited by the controllable application of antisense nucleic acids. An "antisense" nucleic acid as used herein refers to a nucleic acid capable of hybridizing to a sequence-specific (e.g., non-poly A) portion of the target RNA, for example its translation initiation region, by virtue of some sequence complementarity to a coding and/or non-coding region. The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded, RNA or DNA or a modification or derivative thereof, which can be directly administered in a controllable manner to a cell or which can be produced intracellularly by transcription of exogenous, introduced sequences in controllable quantities sufficient to perturb translation of the target RNA.

Preferably, antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 200 oligonucleotides).

As discussed above, antisense nucleotides can be delivered to cells which express the described genes *in vivo* by various techniques, e.g., injection directly into the kidney tissue site, entrapping the antisense nucleotide in a liposome, by administering modified antisense nucleotides which are targeted to the kidney cells by linking the antisense nucleotides to peptides or antibodies that specifically bind receptors or antigens expressed on the cell surface.

However, with the above-mentioned delivery methods, it may be difficult to attain intracellular concentrations sufficient to inhibit translation of endogenous mRNA. Accordingly, in an alternative embodiment, the nucleic acid comprising an antisense nucleotide sequence is placed under the transcriptional control of a promoter, i.e., a DNA sequence which is required to initiate transcription of the specific genes, to form an expression construct. The antisense nucleic acids of the invention are controllably expressed intracellularly by transcription from an exogenous sequence. If the expression is controlled to be at a high level, a saturating perturbation or modification results.

In conclusion, antisense nucleic acids can be routinely designed to target virtually any mRNA sequence including the marker genes (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) citated in the present document, and a cell can be routinely transformed with or exposed to nucleic acids coding for such antisense sequences such that an effective and controllable or saturating amount of the antisense nucleic acid is expressed. Accordingly the translation of virtually any RNA species in a cell can be modified or perturbed.

Small Molecule Drugs or Ligands

In addition, the activities of marker proteins (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) can be modified or perturbed in a controlled or a saturating manner by exposure to exogenous drugs or ligands. Since the methods of this invention are often applied to testing or confirming the usefulness of various drugs to treat kidney disorders, drug exposure is an important method of modifying/perturbing cellular constituents, both mRNA's and expressed proteins.

In a preferable case, a drug is known that interacts with only one marker protein in the cell and alters the activity of only that one marker protein, either increasing or decreasing the activity. Graded exposure of a cell to varying amounts of that drug thereby causes graded perturbations of network models having that marker protein as an input. Saturating exposure causes saturating modification/perturbation.

Antibodies and Antagonists

The term "antagonist" refers to a molecule which, when bound to the protein encoded by the gene, inhibits its activity. Antagonists can include, but are not limited to, peptides, proteins, carbohydrates, and small molecules.

In a particularly useful embodiment, the antagonist is an antibody specific for the markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) . The antibody alone may act as an effector of therapy or it may recruit other cells to actually effect cell killing.

Treatment Modalities

In the case of treatment with an antisense nucleotide, the method comprises administering a therapeutically effective amount of an isolated nucleic acid molecule comprising an antisense nucleotide sequence derived from at least one marker identified in Table 1 above wherein the antisense nucleotide has the ability to change the transcription/translation of the at least one gene.

In the case of treatment with an antagonist, the method comprises administering to a subject a therapeutically effective amount of an antagonist that inhibits or activates a protein encoded by at least one marker identified in Table 1 above.

A "therapeutically effective amount" of an isolated nucleic acid molecule comprising an antisense nucleotide, nucleotide sequence encoding a ribozyme, double-stranded RNA, or antagonist, refers to a sufficient amount of one of these therapeutic agents to treat renal disease or toxicity. The determination of a therapeutically effective amount is well within the capability of those skilled in the art. For any therapeutic, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models, usually rats, mice, rabbits, dogs or pigs. The animal model may also be used to determine the appropriate concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutically effects is the therapeutic index, and it can be expressed as the ratio LD50/ED50. Antisense nucleotides, ribozymes, double-stranded RNAs and antagonists that exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range, depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors that may be taken into account include the severity of the disease state, general health of the subject, age, weight and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy.

Normal dosage amounts may vary form 0.1 to 100,000 micrograms, up to a total dosage of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for antagonists.

For therapeutic applications, the antisense nucleotides, nucleotide sequences encoding ribozymes, double-stranded RNAs (whether entrapped in a liposome or contained in a viral vector) and antibodies are preferably administered as pharmaceutical compositions containing the therapeutic agent in combination with one or more pharmaceutically acceptable carriers. The compositions may be administered alone or in combination with at least one other agent, such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose and water. The compositions may be administered to a patient alone or in combination with other agents, drugs or hormones.

The pharmaceutical compositions may be administered by a number of routes including, but not limited to, oral, intravenous, intramuscular, intra-articular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means. In addition to the active ingredient, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

References cited

All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. In addition, all GenBank accession numbers cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each such number was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatus within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications and variations are intended to fall within the scope of the appended claims. The present invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA are used. These techniques are well known and are explained in, for example, Current Protocols in Molecular Biology, Volumes I, II, and III, 1997 (F. M. Ausubel ed.); Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; DNA Cloning: A Practical Approach, Volumes I and II, 1985 (D. N. Glover ed.); Oligonucleotide Synthesis, 1984 (M. L. Gait ed.); Nucleic Acid Hybridization, 1985, (Hames and Higgins); Transcription and Translation, 1984 (Hames and Higgins eds.); Animal Cell Culture, 1986 (R. I. Freshney ed.); Immobilized Cells and Enzymes, 1986 (IRL Press); Perbal, 1984, A Practical Guide to Molecular Cloning; the series, Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); and Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively).

**Experimental work 1**

Rat kidney samples were obtained from a 2-weeks *in vivo* study conducted in order to identify markers of toxicity and efficacy for immunosuppressants including cyclosporin A (CsA; Neoral®). Neoral® is the reference compound for immunosuppression in terms of clinical applications but also in terms of research model. CsA inhibits early events after T-cell activation, blocking the transcriptional activation of several cytokines.

The kidney, as the major target for toxicity, was studied and the RNA expression changes were monitored in all the groups using the high density DNA-array system from Affymetrix. The in-life part of the study was conducted as follows:

<u>Experimental animals</u>

| | |
|---|---|
| Animal species and strain: | Rats, Crl:WI (GLX/BRL/HAN) IGS BR. |
| Number of animals per group: | 6 males |
| Age: | 8 weeks (at start of dosing). |
| Body weight range: | 100 to 300 g (at start of dosing). |
| Room relative humidity: | Approximately 40 to 70% (target range). |
| Lighting cycle: | Fluorescent light for a 12-hour light/12-hour dark cycle. |
| Animal caging: | Animals were gang housed in groups of the same sex in type IV Macrolon® cages on sterilized softwood particle bedding (manufactured by Rettenmaier & Söhne, Ellwangen-Holzmühle, Germany) under optimal hygienic conditions. |
| Food: | NAFAG, No. 890 pelleted standard diet from NAFAG, Gossau, SG, Switzerland *ad libitum* (batches were given in the on-line raw data), except overnight before blood sampling for clinical pathology. |
| Analysis of food: | Microbiological contaminants investigated by the supplier, chemical contaminants by supplier and RCC Ltd., Environmental Chemical/Pharmaceutical Analytics, Itingen, Switzerland. |
| Water: | Tap water from the local supply was available *ad libitum* from polyethylene bottles. |
| Analysis of water: | Chemical and bacteriological contaminants investigated periodically during the year by municipal authorities and RCC Ltd., Environmental Chemical/Pharmaceutical Analytics, Itingen, Switzerland for compliance with Swiss drinking water specifications. |

The CsA concentration applied in the study was:

Groups : 1: Control; 2: Treatment.

Test item: Neoral ® -Sandimmun (CsA): Dosage (mg/kg): 5; Vol.-dos. (mL/kg): 5.

After the treatment period, kidneys of rats were collected and total RNA was extracted. Total RNA was extracted from frozen kidneys using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Total RNA was quantified by the absorbance at $\lambda$ = 260 nm ($A_{260nm}$), and the purity was estimated by the ratio $A_{260nm}/A_{280nm}$. Integrity was checked by denaturing gel electrophoresis. RNA was stored at -80°C until analysis.

Good quality total RNA was used to synthesize double-stranded cDNA using the Superscript Choice System (Life Technologies). The cDNA was then *in vitro* transcribed (MEGAscript™ T7 Kit, Ambion) to form biotin labeled cRNA. Next, 12 to 15 µg of labeled cRNA were hybridized to the probe arrays for 16 hours at 45°C. Arrays were then washed according to the EukGE-WS2 protocol (Affymetrix), and stained with 10 µg/ml of streptavidin-phycoerythrin conjugate (Molecular Probes). The signal was antibody amplified with 2 mg/ml acetylated BSA (Life Technologies), 100 mM MES, 1 M [Na+], 0.05 % Tween 20, 0.005 % Antiofoam (Sigma), 0.1mg/ml goat IgG and 0.5 mg/ml biotinylated antibody and re-stained with the streptavidin solution. After washing, the arrays were scanned twice with the Gene Array® scanner (Affymetrix).

After mining of the genomics data and under the experimental conditions used in each of the groups, several genes were found to be differentially expressed > 2-fold on each of the probe arrays and were selected for Real-Time PCR confirmation. The GeneSpring™ software was used to compare the expression level in the treatment groups and to sort the genes using clustering algorithms. These calculations separate the genes according to their expression variations and group the genes sharing a similar variation pattern (hierarchical clustering, K-means clustering). It also compares the distribution of the expression level in a specified group to the overall distribution and calculates the probability for a given group to belong to the overall distribution. Genes for which expression changes correlated with the pathological grading were selected. The five gene markers Calbindin-D28k, KIM-1, OPN, EGF and Clusterin constitute part of the specific profile observed on the DNA-arrays after treatment with this agent.

The primer sequences listed in Table 2 have been used for real-time quantitative PCR analysis.-

Table 2: Primer and probe sequences used for the real-time quantitative PCR analysis

| Gene description | Primer name | primer sequence |
|---|---|---|
| Kidney injury molecule 1 (KIM-1) | rKIM1.forward | 5'- CAC TCC ACT TCT GTC TTG ATG CTC -3' |
| | rKIM1.reverse | 5'- GCA CGT CTC CTC CCT GCA -3' |
| | rKIM1.probe | FAM5'- TGT TCC TAA ACT CAC CCA CTG AGC TCT GAA TT -3'TAMRA |
| Calbindin-D28k | rCABP28.forward | 5'-ACA CTG TTG GTT CAA GCT GGC-3' |
| | rCABP28.reverse | 5'-CTT GGA AAT ATA GGC ATA GTA TCA GAC AGA T-3' |
| | rCABP28.probe | FAM5'-TGG TGG CAA GGG AAG GTA GCC AGA-3'TAMRA |
| Osteopontin | rOSTEO.forward | 5'-GAC AGT CAG GCG AGT TCC AAA-3' |
| | rOSTEO.reverse | 5'- CTT GTC CTC ATG GCT GTG AAA C -3' |
| | rOSTEO.probe | FAM5'- CCA GCC TGG AAC ATC AGA GCC ACG -3'TAMRA |
| Epidermal growth factor precursor | rEGFp.forward | 5'- GCA CGA CAT CAC TGT GGT GTC -3' |
| | rEGFp.reverse | 5'- ATC CCC AAG AGG AGC AGC A -3' |
| | rEGFp.probe | FAM5'- TCT GTG TGG TGG CGC TGG CC -3'TAMRA |
| Clusterin | rTRMP2.forward | 5'- AAG GAG GGA ATC TCC CAG CTT -3' |
| | rTRMP2.reverse | 5'- GCG CTG GAG ACA TGT GGA GT-3' |
| | rTRMP2.probe | FAM5'- CCG AGG TTG CTG CAG ACC CCT AGA-3'TAMRA |

Clusterin may be of particular interest as a marker since the product of this gene is a secreted protein. The clusterin protein level was indeed increased as confirmed by Western Blot analysis of serum samples of these animals (Figure 1).

Figure 2 represents the evolution of the expression changes (Fold variation) of the gene markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) linked to kidney tubular basophilia. For comparison the evolution of creatinine excretion (a classical marker) is shown on the Figure 2 in order to demonstrate that the new gene markers (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) described in the present document are more affected and therefore more tightly associated to renal toxicity and are consequently more relevant and valuable.

**Experimental work 2:**

A nonclinical study was initiated to establish the toxicologic effects of a test compound (TC1) when administered to rats for 14 and 42 days. The study was designed to evaluate the potential renal toxicity of the test compound.

The test compound was administered orally by gavage as a solution in Neoral® Placebo Microemulsion Preconcentrate to groups of IGS Wistar Hannover [Crl:WI(Glx/BRL/Han)IGS BR] rats (N=5/sex/group) at doses of 20mg/kg/day, 60mg/kg/day for 14 and at doses of 10mg/kg/day, 25mg/kg/day for 42 days. An additional group of rats (males) received vehicle (Neoral® Placebo Microemulsion Preconcentrate) at an equivalent dosing volume of 5 mL/kg and served as controls. At the initiation of dosing, the animals were approximately 8 weeks of age. Kidney samples were collected at the day of necropsy.

Were monitored by PCR the genes described in the present invention (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) that together or alone, were used as markers of renal toxicity and therefore allowed the evaluation of the nephrotoxicity of the test compound.

Based solely on the expression monitoring of the genes described in the present invention (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin), it could be concluded prior to the pathological examination of kidney microscope slides that the test compound was less nephrotoxic than Neoral (20 mg/kg/day), but slightly damaging for the kidney as demonstrated by the expression of the five gene makers. The kidney toxicity appeared

however to be specific to the test compound after comparison with the Neoral® expression profile. Furthermore, after treatment for 42 days at the dose of 10 mg/kg/day, the gene expression profile for the genes described in the present invention (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) did not indicate marked kidney toxicity (Figure 4). In the figure the "fold-changes Vs control" represent the number of molecules for the genes described in the present invention (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) in the treated groups devided by the number of molecules for the genes described in the present invention (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin) in the respective control groups.

These conclusions were confirmed at a later stage and proved the validity of the prediction made by the monitoring of the expression of the genes described in the present invention (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin). The test compound renal toxicity was caratcterized as being tubular cytoplasmic vacuolation (which was different from the Neoral®-induced renal toxicity as predicted earlier by monitoring the genes described in the present invention (Calbindin-D28k, KIM-1, OPN, EGF and Clusterin).

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**We Claim**:

1. A method for determining renal toxicity in an individual comprising:

   (a) obtaining a body sample from an individual,

   (b) determining from the body sample the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value, and

   (c) comparing the first set of value with a second set of value corresponding to the level of gene expression assessed for the same gene(s) and under identical condition as for step b) in a body sample of an individual not subject to renal toxicity, wherein the first value lower than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the individual of step a) is having, developing or sensitive to renal toxicity, and/or wherein the first value greater than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the individual is having, developing or sensitive to renal toxicity.

2. The method of claim 1, wherein in steps b) and c) at least 2 or 3 genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin are used.

3. The method of claim 1 or 2, wherein mRNA expression levels in the body sample of the individual of step a), of Calbindin-D28k below 5.30E+08, of KIM-1 above 1.50E+07, of EGF below 2.80E+08, of Osteopontin above 1.40E+08, and/or of Clusterin above 1.90E+09, indicates that such individual is having, developing or sensitive to renal toxicity, wherein mRNA expression levels are expressedin absolute value.

4. The method of claims 1 to 3 wherein the level of gene expression is assessed by detecting the presence of a protein corresponding to the gene expression product.

5. A test for use in determining whether a renal toxicity in an individual will respond to therapy comprising the steps of, performing steps a), b) and c) set forth in one of the claims 1 to 4 for a body sample obtained from an individual treated against renal toxicity with a pharmaceutically acceptable agent and determining the responsiveness of the individual to drug therapy.

6. A method for treating renal toxicity in an individual comprising the step of administering to said individual a therapeutically effective amount of a modulating compound that

modulates in the kidney the synthesis, expression or activity of one or more of the genes or gene expression products of the group of genes Calbindin-D28k, KIM-1, OPN, EGF and/or Clusterin, so that at least one symptom of renal toxicity is ameliorated.

7. A method of claim 6, wherein after treatment with the modulating compound the renal toxicity of the individual is determined according to claims 1-4, and wherein gene mRNA expression levels in a body sample of the individual, of Calbindin-D28k above to 5.30E+08, of KIM-1 below 1.50E+07, of EGF above 2.80E+08, of Osteopontin below 1.40E+08, and/or of Clusterin below 1.90E+09, indicates that at least one symptom of renal toxicity is ameliorated, wherein gene mRNA expression levels are expressed in absolute value.

8. A method for identifying candidate agents for use in the treatment of renal toxicity comprising the steps of:

a) contacting a sample of a kidney tissue subject to toxicity with a candidate agent;

b) determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value, and

c) comparing the first set of value with a second set of value corresponding to the level of gene expression assessed for the same gene(s) and under identical condition as for step b) in a kidney tissue subject to toxicity not induced by the candidate agent wherein a first value substantially greater than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the candidate agent is ameliorating renal toxicity symptoms, and/or wherein a first value substantially lower than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the candidate agent is ameliorating renal toxicity symptoms.

9. The method of claim 8, wherein in steps b) and c) at least 2 or 3 genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin are used.

10. The method of claim 8 or 9, wherein mRNA gene expression level in kidney tissue subject to toxicity, of Calbindin-D28k above 5.30E+08, of KIM-1 below 1.50E+07, of EGF above 2.80E+08, of Osteopontin below 1.40E+08, and/or of Clusterin below 1.90E+09, is

an indication that the candidate agent is ameliorating renal toxicity, wherein mRNA gene expression level is expressed in absolute value.

11. The method of claims 8 to 10 wherein the level of gene expression is assessed by detecting the presence of a protein corresponding to the gene expression product.

12. A method for identifying candidate agents that do not provoke or induce renal toxicity comprising the steps of:

a) contacting a sample of a kidney tissue not subject to toxicity with a candidate agent,

b) determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of values, and

c) comparing the first set of value with a second set of value corresponding to the level of gene expression assessed for the same gene(s) and under identical condition as for step b) in a kidney tissue not subject to toxicity, wherein a first value equal or higher than the second value for Calbindin-D28K and/or EGF gene expression is an indication that the candidate agent does not provoke or induce renal toxicity, and/or wherein a first value equal or lower than the second value for KIM-1, Osteopontin and/or Clusterin gene expression is an indication that the candidate agent does not provoke or induce renal toxicity.

13. The methods of claim 12, wherein in steps b) and c) at least 2 or 3 genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin are used. 14. The method of claim 12 or 13, wherein mRNA expression level determined in the kidney tissue not subject to toxicity, of Calbindin-D28k above 5.30E+08, of KIM-1 below 1.50E+07, of EGF above 2.80E+08, of Osteopontin below 1.40E+08, and/or of Clusterin below 1.90E+09, is an indication that the candidate agent does not provoke or induce renal toxicity, wherein mRNA expression level is expressed in absolute value.

15. The method of claims 12 to 14 wherein the level of gene expression is assessed by detecting the presence of a protein corresponding to the gene expression product.

16. The method of claims 8 to 15 wherein the method is performed *in vitro*, and the kidney tissue subject to toxicity is obtained from a cultured kidney tissue contacted with a cytotoxic agent under cytotoxic conditions. 17. The method of claims 8 to 15 wherein the kidney tissue subject to toxicity is a kidney sample of an individual subject to renal toxicity, said sample having mineralization, fibrosis, tubular, infiltration, necrosis damages or any other kind of damages that results in renal dysfunction.

18. A method for comparing renal cytotoxic potentials of two drug candidates comprising the steps of:

a) contacting a sample of a kidney tissue not subject to toxicity with a first drug candidate, and determining from the kidney tissue the level of gene expression corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a first set of value, and

a) contacting a sample of a kidney tissue not subject to toxicity with a second drug candidate, and determining from the kidney tissue level(s) of gene expression(s) corresponding to one or more genes selected among Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, to obtain a second set of value, and

c) comparing the first set of value to the second set of value, wherein if the first value is substantially lower than the second value for Calbindin-D28K and/or EGF gene expression this is an indication that the second drug candidate is less cytotoxic to the kidney than the second drug candidate, and/or wherein if the first value is substantially higher than the second value for KIM-1, Osteopontin and/or Clusterin gene expression this is an indication that the second drug candidate is less cytotoxic to the kidney than the second drug candidate.

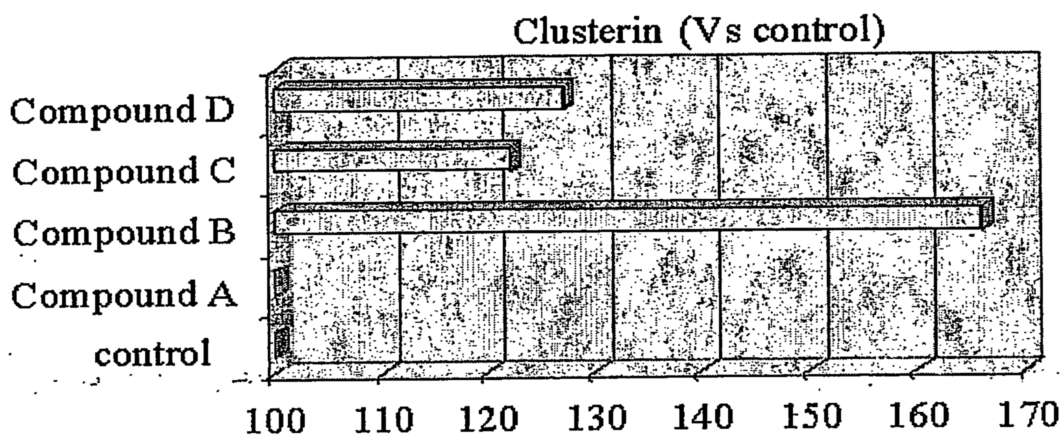
19. The method according to the preceeding claims wherein the level of expression of mRNA is detected by techniques selected from the group consisting of Northern blot analysis, reverse transcription PCR and real time quantitative PCR, branched DNA, nucleic acid sequence based amplification (NASBA), transcription-mediated aplification, ribonuclease protection assay, or any other methods for gene expression analysis currently available or that are to come

20. The use of some polymorphism in a gene for the diagnostic of renal toxicity, wherein the gene are chosen from Calbindin-D28k, KIM-1, OPN, EGF and Clusterin.

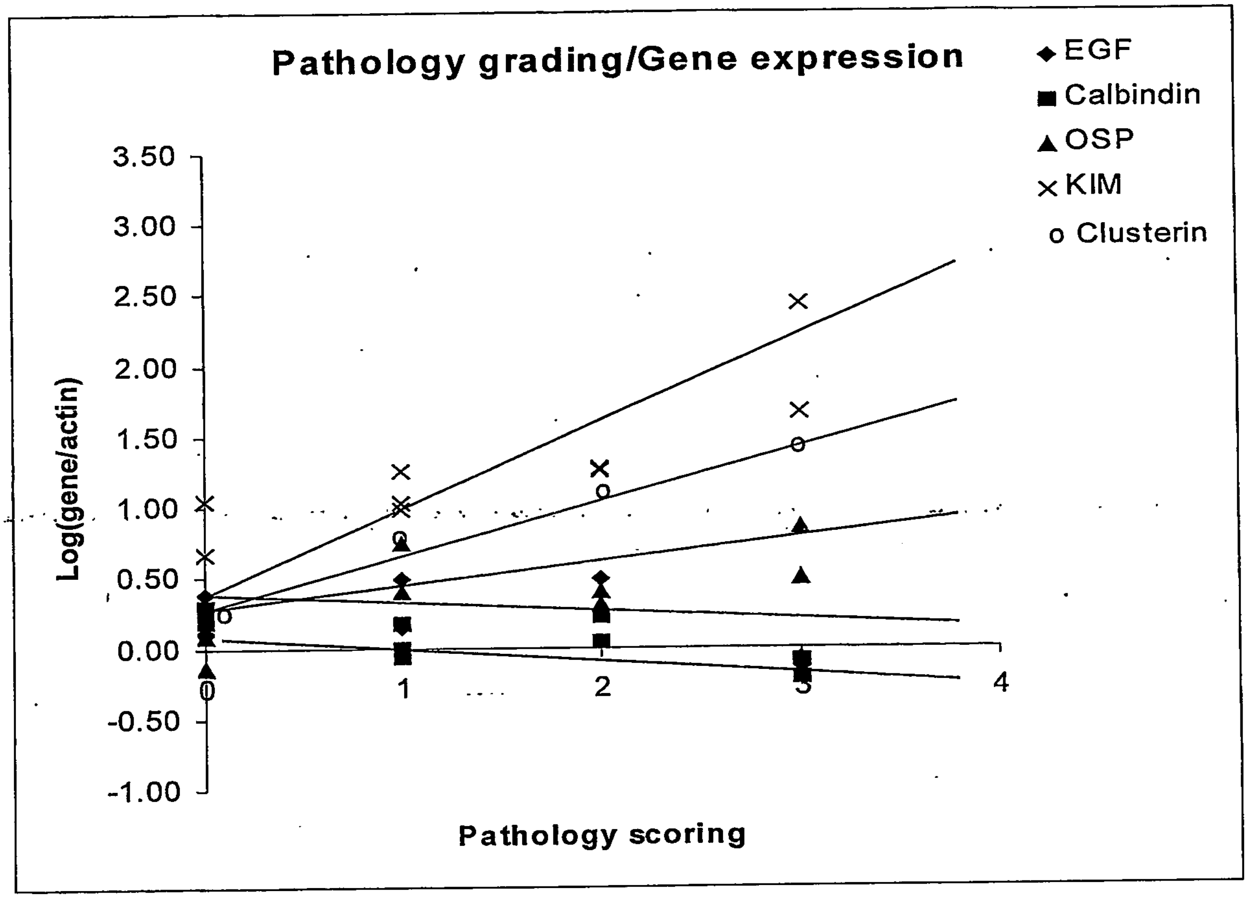
## ABSTRACT

Methods are disclosed for fast and accurate readout of kidney toxicity before it occurs and before it is demonstrated by histopathology examination. Ultimately this approach shall allow earlier compound selection. The five genes identified, namely Calbindin-D28k, KIM-1, OPN, EGF and Clusterin, were grouped and ultimately can be assessed in the form of a kit using PCR, a high throughput technology, in order to characterize and rank new compounds according to their anticipated general kidney toxicity. Also disclosed are methods for identifying agents useful in the treatment of kidney disease, methods for monitoring the efficacy of a treatment for kidney disease and kidney-specific vectors including the sequences of the disclosed genes.

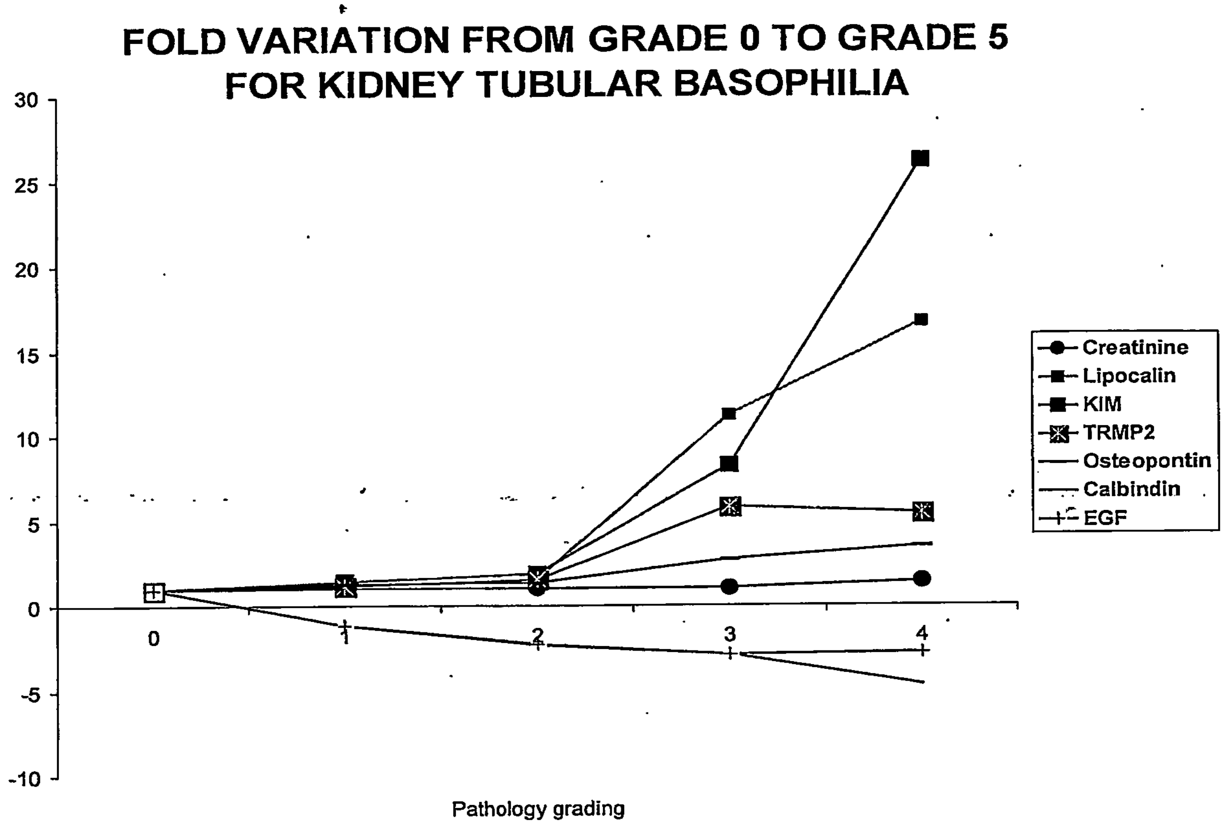
**1/4**

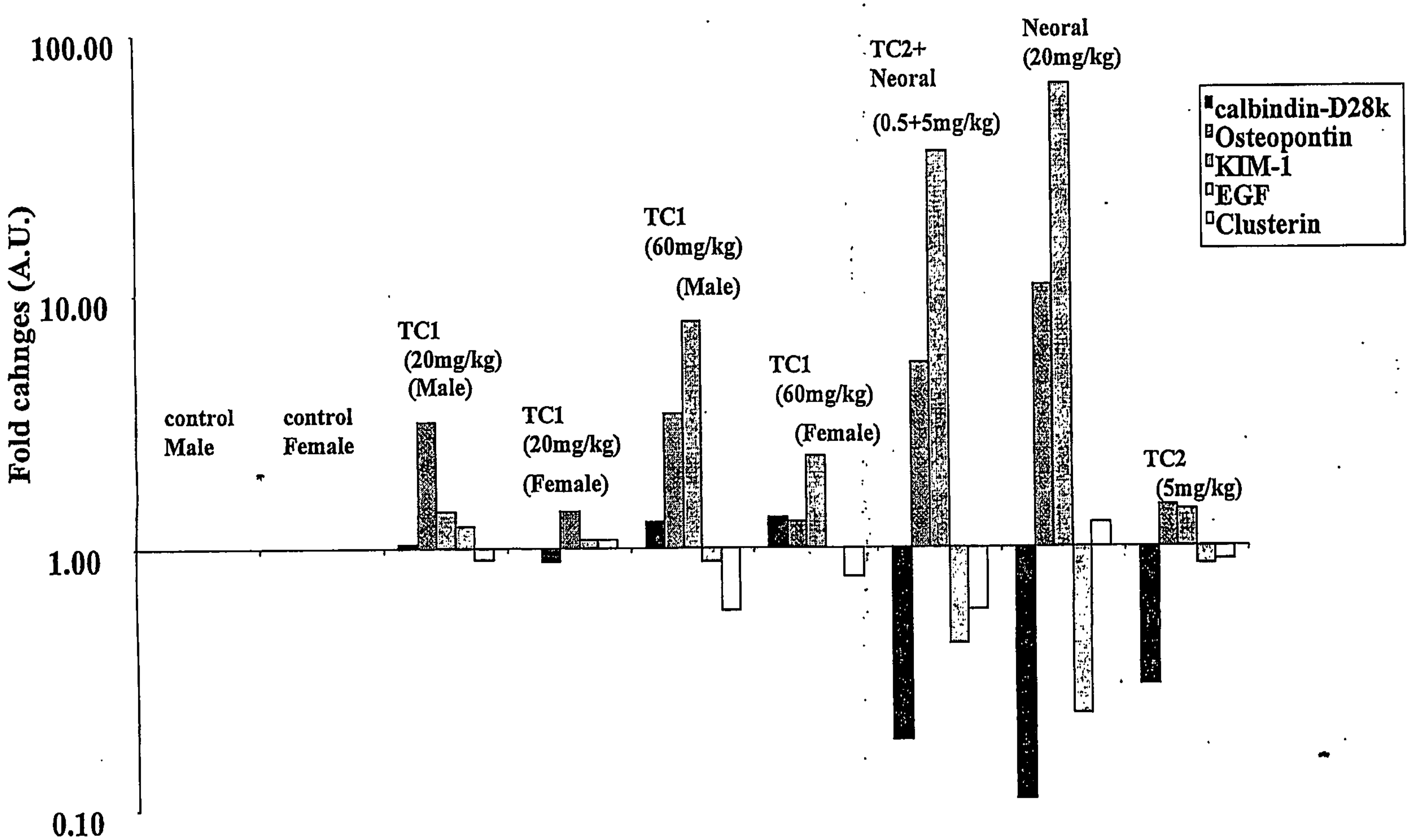

Clusterin (Vs control)
Compound D
Compound C
Compound B
Compound A
control
100
110
120
130
140
150
160
170

2/4


Pathology grading/Gene expression
EGF
Calbindin
OSP
KIM
Clusterin
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
Log(gene/actin)
0
1
2
3
4
Pathology scoring

3/4


FOLD VARIATION FROM GRADE 0 TO GRADE 5
FOR KIDNEY TUBULAR BASOPHILIA
30
25
20
15
10
5
0
-5
-10
0
1
2
3
4
Pathology grading
Creatinine
Lipocalin
KIM
TRMP2
Osteopontin
Calbindin
EGF



Fold cahnges (A.U.)
100.00
10.00
1.00
0.10
control Male
control Female
TC1 (20mg/kg) (Male)
TC1 (20mg/kg) (Female)
TC1 (60mg/kg) (Male)
TC1 (60mg/kg) (Female)
TC2+ Neoral (0.5+5mg/kg)
Neoral (20mg/kg)
TC2 (5mg/kg)
calbindin-D28k
Osteopontin
KIM-1
EGF
Clusterin